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Award Number:
W81XWH-06-1-0515

TITLE:
Molecular determinants of estrogen receptor alpha stability

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CONTRACTING ORGANIZATION:
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REPORT DATE:
July 2008

TYPE OF REPORT:
Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2008	Annual Summary	1 JUL 2007 - 30 JUN 2008

4. TITLE AND SUBTITLE
Molecular determinants of estrogen receptor alpha stabilstability

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0515

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Carolyn DuSell

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University, Durham, NC 27710

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Material Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We have identified a novel endogenous ligand for the estrogen receptor, that being 27-hydroxycholesterol (27HC). 27HC mimics the effects of estrogen when assayed on multiple endpoints, including target gene regulation, inducing receptor turnover in an AIB1-dependent manner, and increasing breast cancer cell proliferation. Current studies are now focused on two main areas. First, we aim to determine whether macrophages produce 27HC in sufficient quantities to affect breast cancer cell behavior. Infiltrating macrophages are associated with reduced survival from breast cancer, and we hypothesize that one explanation for this is local production of 27HC, which acts as a mitogen. Second, we are interested in identifying proteins that bind specifically to 27HC-bound ER, and then to ascertain the biological significance of these proteins as they impact ER signaling. Previous studies had determined that levels of 27HC are positively correlated to that of cholesterol. Given the current epidemic of obesity/hypercholesterolemia, our studies on the impact of 27HC on ER are crucial for our understanding of how the physiological impact of this epidemic.

15. SUBJECT TERMS
Estrogen receptor alpha, 27-hydroxycholesterol

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	11	**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

The estrogen receptor (ER) is critically important for the development and maintenance of many tissues, including the reproductive system, the cardiovascular system, and the skeleton. The most prevalent endogenous estrogen during a woman's reproductive years is 17β-estradiol (E2). The actions of E2 are mediated primarily through binding to either of the two isoforms of ER, ERα and ERβ (*1, 2*). In the nucleus, this binding induces a conformational change in ER that leads to receptor homo- or hetero-dimerization, DNA binding, recruitment of coregulatory proteins, and the modulation of target gene transcription.

Besides their important normal physiological roles, estrogens also are a major contributor to the growth and progression of ER-positive breast cancer. This has provided an excellent target for the development of therapeutics for the treatment of ER-positive breast cancer, the anti-estrogens. With the discovery of tamoxifen, researchers quickly realized that not all anti-estrogens had the same activity profiles, nor did they antagonize ER signaling in all tissues. Rather, they differed in terms of tissue-specific agonist and antagonist activities, leading to the re-classification of some anti-estrogens, such as tamoxifen and raloxifene, as Selective Estrogen Receptor Modulators (SERMs). This elicited the question as to whether these SERMs mimic endogenous molecules with similar properties or resulted from a serendipitous ability to selectively manipulate the canonical estrogen signaling axis. The recent discovery of 27-hydroxycholesterol (27HC) as an endogenous ER ligand with potential tissue-specific agonist and antagonist actions begins to answer this question.

27HC is an oxysterol, a derivative of cholesterol that is involved in regulating intracellular cholesterol homeostasis. The action of the P450 enzyme CYP27A1 produces 27HC from cholesterol, and further hydroxylation by CYP7B1 leads to the metabolism of 27HC. As 27HC is more polar than cholesterol, it can exit the cell and travel through the circulation to the liver, where it can enter further into the bile acid synthesis pathway. As of now, it is unclear whether 27HC regulates ER signaling by acting in an intracrine, paracrine, or endocrine manner. It is now our goal to understand how 27HC regulates ER and what the biological consequences of this regulation are.

Body

In task 2 of our revised statement of work, we set out to characterize 27HC as a novel ER ligand. Our first objective was to determine if 27HC could regulate the transcriptional activity of ERα, as assessed on a classic estrogen response element (ERE) in a system of transfected receptor and reporter. ER-negative cells were transfected with both ERα and the ERE-luc construct, treated with vehicle or increasing concentrations of 27HC or E2, then assayed for luciferase expression. As shown in **Figure 1 (Task 2.1)**, 27HC treatment led to a dose-dependent increase in ERα transcriptional activity. Interestingly, 27HC treatment does not lead to as robust a transcriptional response as E2. Further, when we co-treated cells with a fixed concentration of E2 and increasing concentrations of 27HC, we found that 27HC is able to compete with E2 to decrease the maximal transcriptional activity.

Given our data that 27HC acts as a partial agonist on ERα, we wanted to study the conformational change induced in ERα upon binding of 27HC. Ligand-induced conformational changes are critical in dictating the resulting biological activity by determining which coregulatory proteins can bind to ERα. These conformational changes can be investigated using small peptide probes that will bind to the distinct surfaces presented on ERα in the presence of different ligands. Previously we identified peptides that bind to ERα in the presence of all agonists, of 4-hydroxytamoxifen (4OHT), or of the pure antagonist ICI 182,780 (ICI). Using these peptides in a mammalian 2-hybrid assay, we determined that 27HC induces a conformation that most closely resembles an agonist-induced conformation. As shown in **Figure 2A**, 27HC allows for recruitment of the agonist peptide D30, but not the 4OHT peptide bT1 or the ICI peptide bI2. However, given the differences in transcriptional activation and peptide recruitment between 27HC and E2, we wanted to

identify more peptide probes that would better distinguish between the conformation of ERα induced by 27HC versus E2. To accomplish this, we performed combinatorial phage display using a modified M13 phage display screen. We screened two libraries, one with peptides containing the LxxLL motif and the other with the CoRNR box motif, against DNA-bound ERα in the presence of 27HC. From this screen, we isolated different classes of peptides, with representative ones shown in **Figure 2B (Task 2.2a)**. The majority of the identified peptides bound to ERα in the presence of 27HC or E2, confirming that the conformation induced by 27HC is agonist-like and shares many similarities with that induced by E2. However, we were able to isolate peptides that were quite selective for the 27HC-ERα conformation, and also peptides that shared preference between 27HC and 4OHT, indicating that 27HC-ERα might be able to recruit corepressors, potentialy explaining the lack of complete agonist activity seen with this ligand.

Given the finding that 27HC induces an agonist-like conformation of ERα, but one that is not identical to E2-ERα, we were curious to see if 27HC-ERα could recruit peptides containing the nuclear receptor interacting domains of classic coactivators. This also began our investigation into whether there exist distinct coactivator preferences between 27HC-ERα and E2-ERα. As shown in **Figure 3 (Task 2.2b)**, 27HC- and E2-ERα were both able to bind peptides from SRC1, ASC2, AIB1, and GRIP1 with no clear differences between the two ligands. Further studies are warranted here to more thoroughly investigate coactivator and corepressor recruitment by 27HC-ERα.

Although we have shown that 27HC has agonist activity in transcription assays and induces an agonist-like conformation of ERα, we wanted to determine whether 27HC could lead to increased recruitment of ERα to the promoters of classic target genes, such as pS2, in a manner analogous to E2. In **Figure 4 (Task 2.3)** we show that 27HC treatment of MCF-7 cells led to increased occupancy of ERα at the pS2 promoter, albeit not to the same level as E2 treatment.

Next we sought to establish whether 27HC acts as an agonist in ER-positive breast cancer cells in terms of endogenous target gene regulation. Using MCF-7 and T47D cells, we analyzed gene regulation by quantitative real time PCR (qRT-PCR). Increasing concentrations of 27HC led to dose-dependent regulation of target genes such as SDF-1, PR, pS2, E2F1, WISP2, and ERBB4 in the MCF-7 cells **(Figure 5A (Task 2.4a))**. Again, the response to 27HC was not as robust as that to E2, but the two ligands regulated target genes similarly. Comparable results were obtained in the T47D cell line **(Figure 5B (Task 2.4a))**. Importantly, we were able to show that gene expression in response to 27HC was inhibited by co-treatment with ICI **(Figure 5C (Task2.4b))**.

A hallmark of many ERα agonists is that they induce ligand-dependent degradation of the receptor protein. Interestingly, the degradation induced by E2, for example, is intimately linked to transcriptional activation. Therefore, we examined whether 27HC also leads to degradation of ERα in MCF-7 cells. Treatment with 27HC for 1, 4, 8, or 24 hours showed a time-dependent decrease in protein levels, as shown in **Figure 6 (Task 2.5a)**. Further, we investigated whether the coregulatory protein AIB1 is required for 27HC-dependent ERα degradation, as it is for E2-ERα. Using siRNA technology, we knocked down AIB1 protein levels and assayed ligand-dependent degradation. After 8 hours, 27HC induced significant degradation of ERα only in the presence of AIB1, seen in **Figure 7 (Task 2.5b)**.

Taken together, our data thus far suggests that 27HC mimics the effects of E2 in cellular models of ER-positive breast cancer. The major contribution of E2 in breast cancer is as a mitogen that increases cell proliferation. Therefore, our pivotal study was to ascertain whether 27HC could also mimic E2 in this regard. First, we showed by qRT-PCR that 27HC treatment led to an increase in Cyclin D1 expression, a key regulator of cell proliferation **(Figure 7A (Task 2.6))**. Second, we showed that increasing concentrations of 27HC led to increased BrdU incorporation, a surrogate for the number of cells entering S-phase of the cell cycle, as seen in **Figure 7B (Task 2.6)**. Lastly, 27HC

led to a dose-dependent increase in overall cell proliferation as assessed by an increase in total cell number after 6 days of ligand treatment **(Figure 7C (Task2.6))**.

Thus, 27HC is an endogenous regulator of the ER signaling axis, and in this context mimics many of the actions of E2, albeit with reduced efficacy. The full biological consequences of these actions have yet to be elucidated, but our future studies are aimed at tackling a few basic questions. One, there exists a correlation between infiltrating macrophages and reduced survival from breast cancer, and we are interested in determining if 27HC is somehow involved. Macrophages are great producers of 27HC *in vivo*, and it is possible that the accumulation of macrophages in a breast tumor provides a local estrogenic source, and importantly one that is not dependent on aromatase activity. This can be addressed in part with the use of co-cultures of macrophages and breast cancer cells, as well as the use of spent media from macrophage cultures as a treatment source for proliferation assays and gene expression studies **(Task 3)**. Second, we are interested in identifying unique protein-protein interactions that occur with ERα in the presence of 27HC. Given that the peptide binding studies showed that the conformation induced by 27HC is not the same as that induced by E2, one can speculate that perhaps 27HC allows ERα to interact with proteins that are not capable of binding to this receptor in the presence of E2. This question can be addressed by a T7 phage display screen using a breast cancer cell library and DNA-bound ERα in the presence of 27HC **(Task 4)**.

Key Research Accomplishments

- 27-hydroxycholesterol can recapitulate many of the actions of estradiol in *in vitro* models of ERα-positive breast cancer

Reportable Outcomes

Publications:

DuSell CD and DP McDonnell. 27-Hydroxycholesterol: a potential endogenous regulator of estrogen receptor signaling. Trends in Pharmacological Sciences 29 (10): 510 – 514 (2008).

DuSell CD, Umetani M, Shaul PW, Mangelsdorf DJ, and DP McDonnell. 27-Hydroxycholesterol is an Endogenous Selective Estrogen Receptor Modulator. Molecular Endocrinology 22(1): 65-77 (2008).

Presentations:

DuSell C.D. (Presenter), Umetani M., Shaul P.W., Mangelsdorf D.J., & McDonnell D.P. "27-hydroxycholesterol: an Endogenous Selective Estrogen Receptor Modulator". Department of Pharmacology and Cancer Biology Annual Retreat, September 26-28 2008, Wrightsville Beach, NC (Invited Speaker).

DuSell C.D. (Presenter), Umetani M., Shaul P.W., Mangelsdorf D.J., & McDonnell D.P. "27-hydroxycholesterol is an Endogenous Selective Estrogen Receptor Modulator". Department of Defense Era of Hope Meeting, July 23-26 2008, Baltimore, MD (Poster).

DuSell C.D. (Presenter), Umetani M., Shaul P.W., Mangelsdorf D.J., & McDonnell D.P. "27-hydroxycholesterol is an Endogenous Selective Estrogen Receptor Modulator". Duke University Graduate Student Symposium, Nov. 9 2007, Durham, NC (Poster).

DuSell C.D. (Presenter), Umetani M., Shaul P.W., Mangelsdorf D.J., & McDonnell D.P. "27-hydroxycholesterol is an Endogenous Selective Estrogen Receptor Modulator". Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications (AACR), Oct. 17-20 2007, San Diego, CA (Poster).

DuSell C.D. (Presenter) & McDonnell D.P. "27-hydroxycholesterol is a Novel Endogenous Regulator of Estrogen Receptor Activity". Duke Comprehensive Cancer Center Annual Meeting, Mar. 12 2007, Durham, NC (Poster).

DuSell C.D. (Presenter), Umetani M., Shaul P.W., Mangelsdorf D.J., & McDonnell D.P. "27-hydroxycholesterol is an Endogenous Selective Estrogen Receptor Modulator". Department of Pharmacology and Cancer Biology Annual Retreat, 2007, Wrightsville Beach, NC (Poster).

Conclusion

27-hydroxycholesterol (27HC) is an endogenous regulator of ER signaling that likely has a profound role in controlling cellular responses to estradiol. Current work has elicited a role for 27HC in the cardiovascular system and the breast (*3, 4*). Future studies will continue to characterize the role of 27HC in these tissues, and will also expand our knowledge of this aspect of ER signaling by examining the role of 27HC in other ER-responsive tissues, such as the bone.

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2. G. G. Kuiper, E. Enmark, M. Pelto-Huikko, S. Nilsson, J. A. Gustafsson, *Proc Natl Acad Sci U S A* **93**, 5925 (Jun 11, 1996).
3. C. D. DuSell, M. Umetani, P. W. Shaul, D. J. Mangelsdorf, D. P. McDonnell, *Mol Endocrinol* **22**, 65 (Jan, 2008).
4. M. Umetani *et al.*, *Nat Med* **13**, 1185 (Oct, 2007).

Appendices

None.

Supporting Data

Figure 1

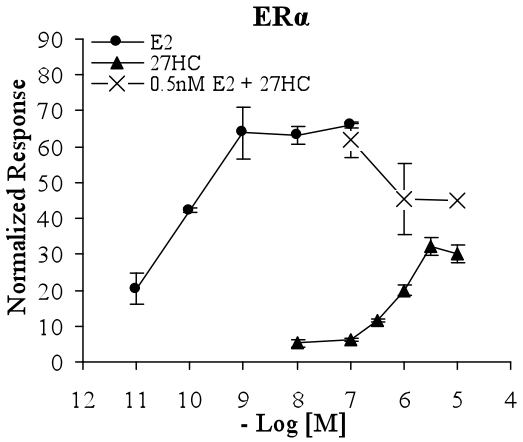

Figure 2

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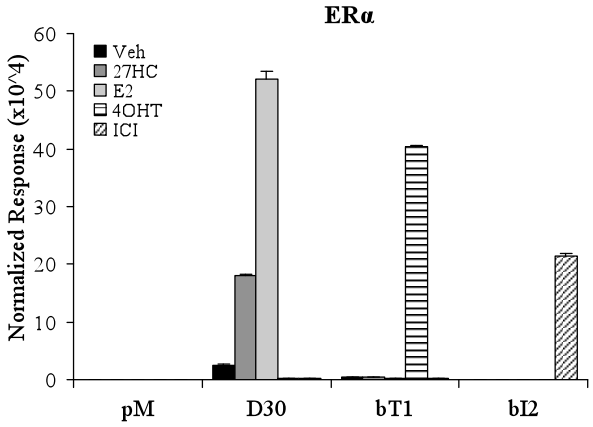

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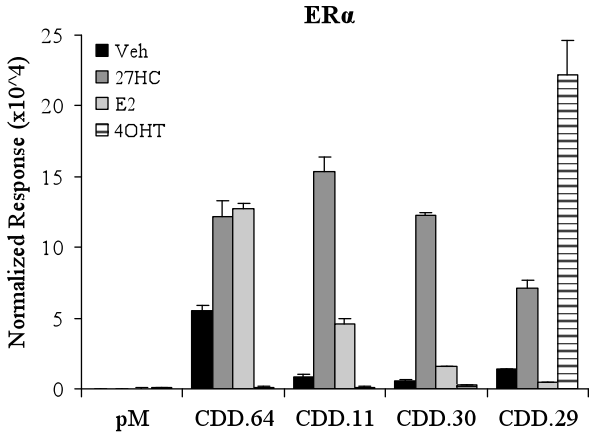

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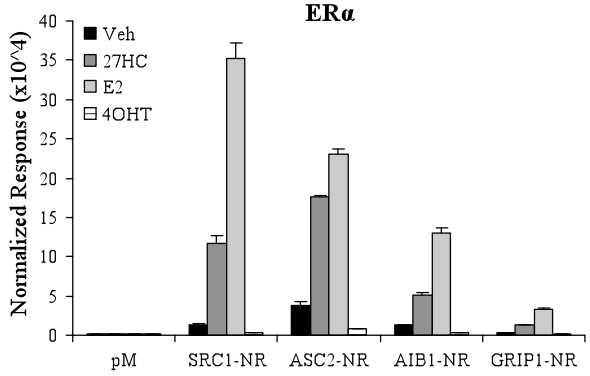

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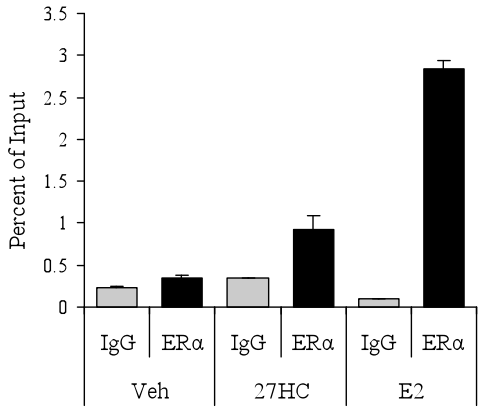

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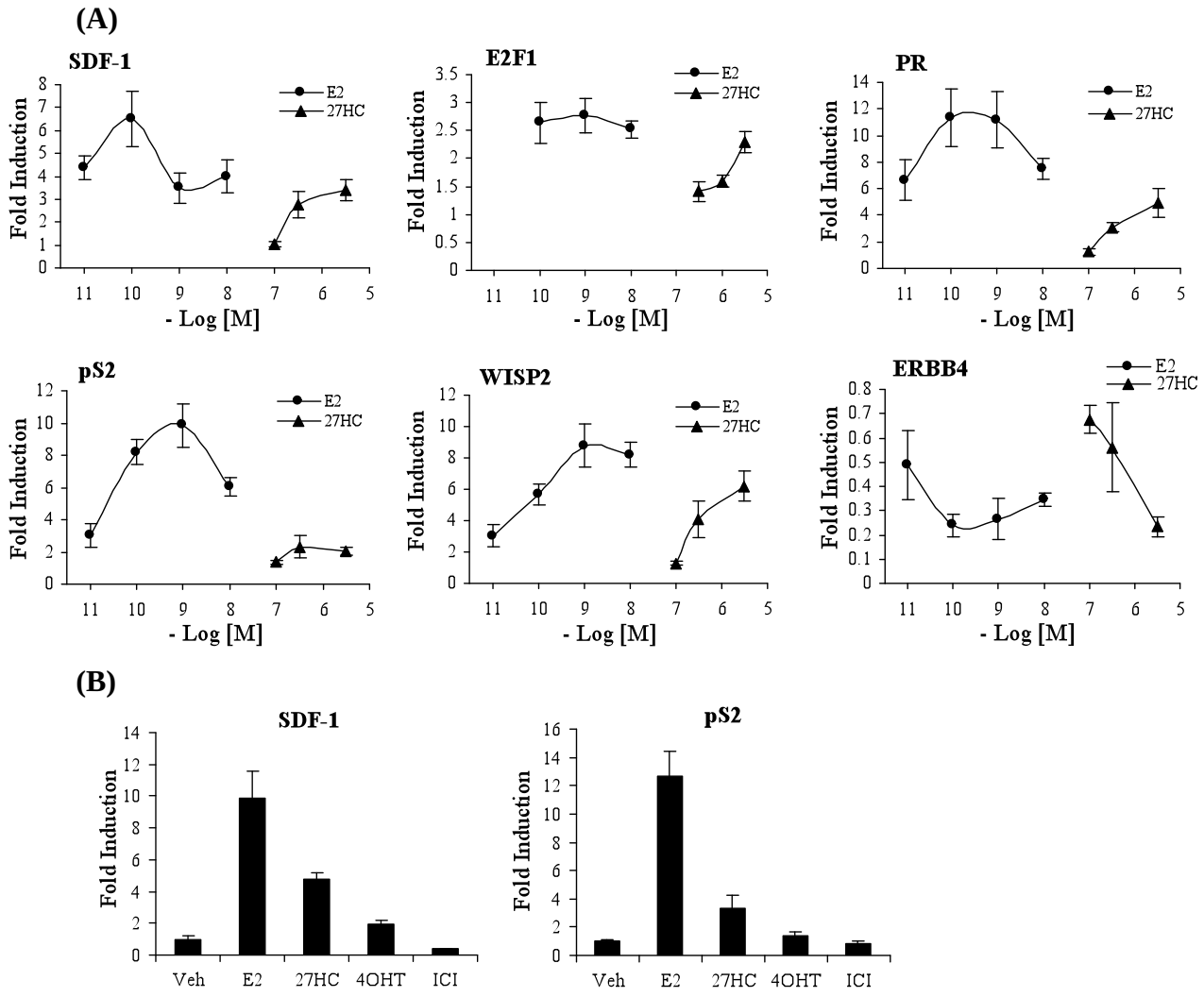

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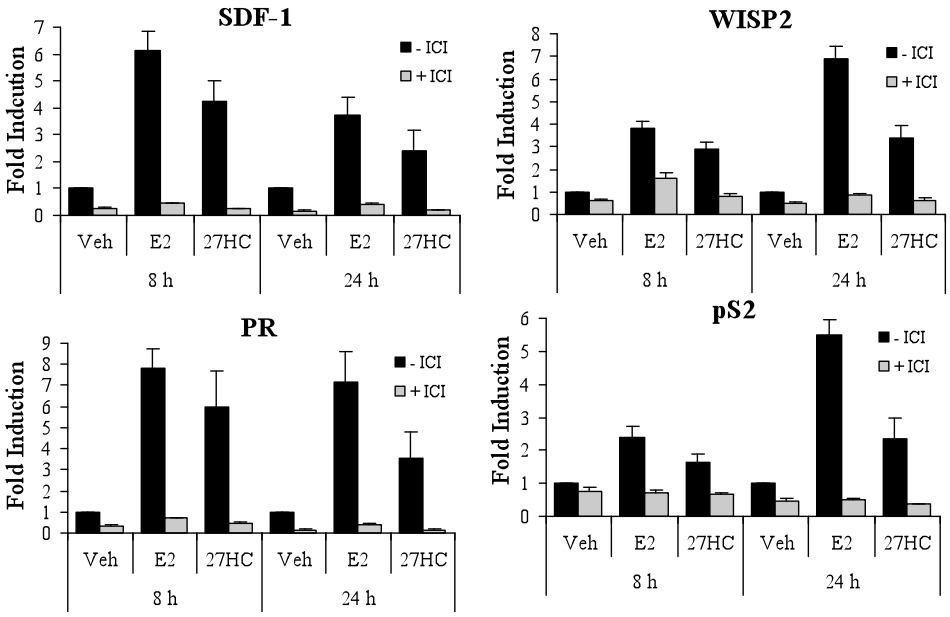

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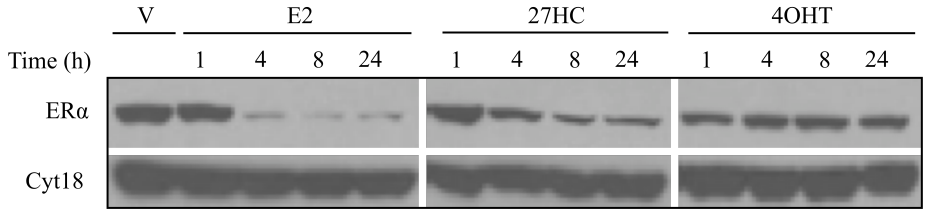

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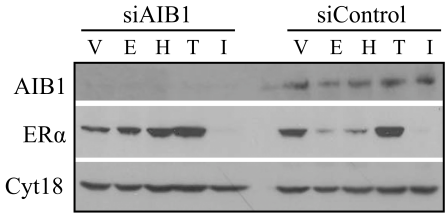

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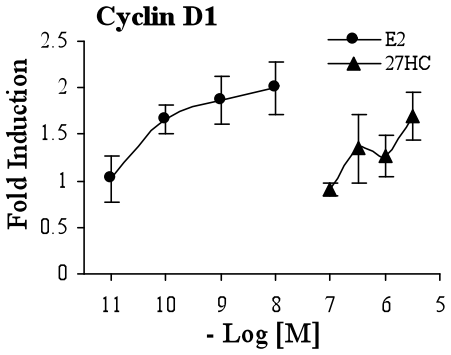

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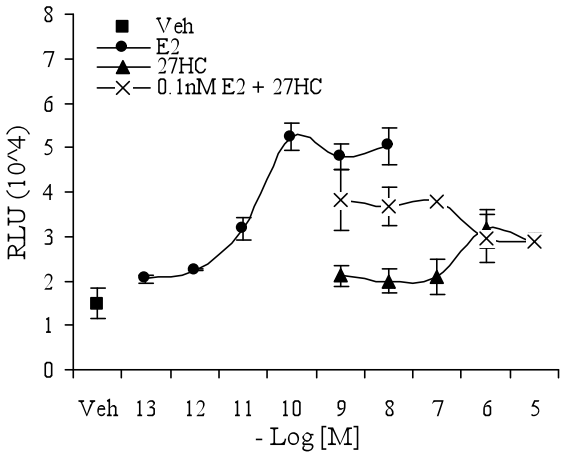
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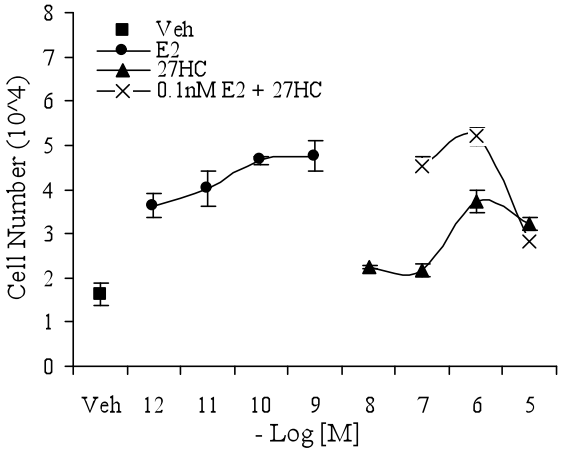
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